Newsletter from the Institute of Genetic Ecology 9

NEWSLETTER 9

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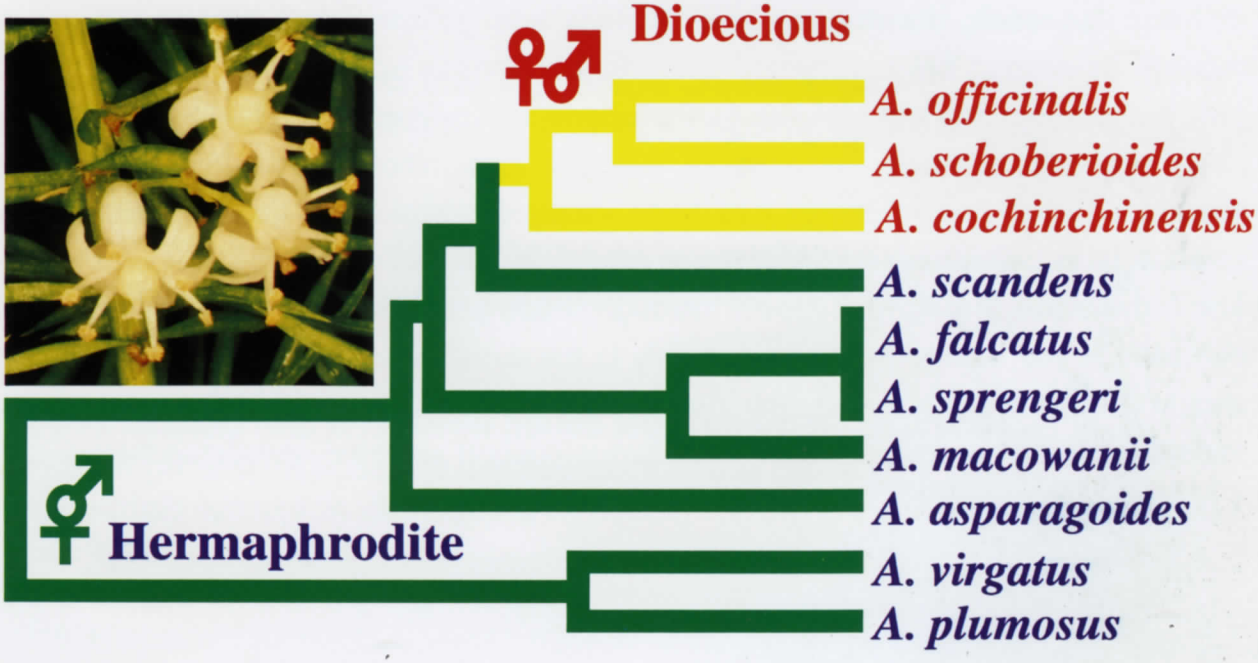
The Institute of Genetic Ecology



TOHOKU UNIVERSITY 1997

PREFACE

With the reorganization of the Institute for Agricultural Research in 1988, the Institute of Genetic Ecology was established with the purpose of studying the genetic basis of species in ecosystems, utilizing the knowledge gained during the era of the previous institute. Through eons of evolution, plants and microorganisms have been able to survive some unfavorable alterations in environmental conditions by means of several strategies, such as avoidance, adaptation and tolerance. Such outwardly small changes in the behavior of plants and microorganisms, however, have the potential to induce alterations in the ecosystem on a global scale. The behavior and responses of the organisms depend upon the diversity of their genetic characteristics. To understand the complex mechanisms of a such dynamic ecosystem, therefore, a diphasic approach, drawing on both ecology and genetics, is essential. Thus, we recently developed the interdisciplinary science, Genetic Ecology. In recent years, not only the atmospheric but also the hydrospheric and pedospheric environments have been seriously polluted. Because of this, the recognition of the importance and imminence of Genetic Ecology has been increasing rapidly. A new function of the institute is to participate in cooperative programs with scientists of other institutions and universities throughout Japan. Through these programs, we are convinced that our institute will further contribute not only to the development of Genetic Ecology but also to greater exploitation of new interdisciplinary sciences. I would like to conclude my remarks with an entreaty for your generous support and kind understanding.

Tamotsu OOTAKI, Director

CONTENTS

Rhizobium-Legume Interactions: Regulation, Perception and Prospects

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Gram negative bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Azorhizobium*, and *Sinorhizobium* are capable of establishing a nitrogen-fixing symbiosis via the infection of roots of their leguminous hosts. This strain-specific infection is mediated by a signal-exchange between the infecting bacterium and the host plant, and results in the development of a novel organ, the nodule. Within the nodule, the bacteria then differentiate into bacteroids, where they reside, fixing atmospheric nitrogen which is provided to the host plant in the form of ammonia. In turn, the host plant provides the bacteria with a source of carbon. Plant-produced flavonoids initiate the establishment of the symbiosis through the induction of the bacterial nodulation genes, which function in the synthesis and transport of lipo-chitin oligosaccharides. These signal molecules, Nod Signals, are oligomers (usually four to five residues) of β-1,4-linked N-acetyl-glucosamine residues and are capable of inducing root deformation, as well as root cortical cell division, when applied to the developing roots. These compounds play a key role in the initiation of nodulation Some evidence even suggests that the specificity of these molecules is due to the presence of specific substitutions to the chitin backbone. Research in our laboratory is focused on understanding the mechanisms of regulation involving the expression of *nod* genes in *Bradyrhizobium japonicum,* as well as characterizing the signal molecules produced by this bacterium. More recently, we have also begun to investigate the role of various plant-produced proteins in the recognition of these signal molecules, and the role these proteins may play in nodulation.

nod Gene Regulation

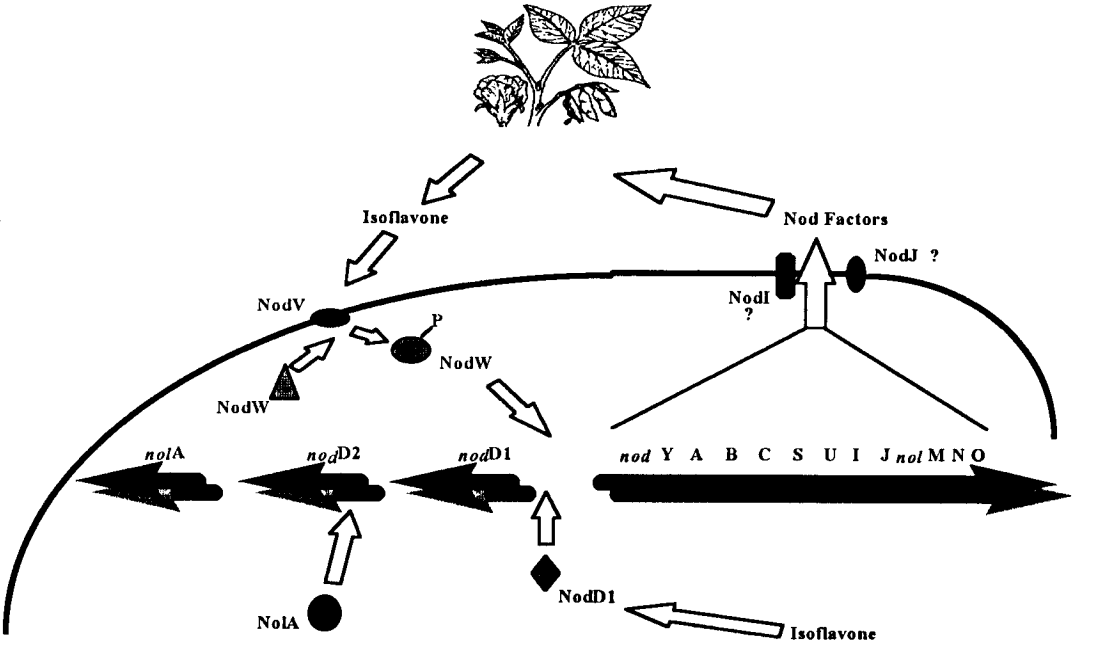
The nodulation (*nod*, *nol*, and *noe*) genes are involved in the synthesis and secretion of Nod Signals. Mutations in these genes can result in the loss of nodulation, the alteration of the range of host-specificity, or specific changes in the structure of the Nod Signal. The

common *nod* genes, *nod*ABC are responsible for the synthesis of the chitin backbone, and mutations in these genes result in the loss of production of Nod Signals. The other *nod* genes are not as well conserved between species. Activation of the *nod* genes appears to be mediated through the involvement of at least two pathways in *B. japonicum*. The first involves the bacterial protein, $NodD_1$, which functions as a transcriptional regulatory protein common to all genera of the Rhizobiaceae family, and is a member of the LysR-family of transcriptional regulators (reviewed in Schell, 1993). Activation of this pathway is believed to be mediated by the interaction of the $NodD_1$ protein with the plant-produced flavonoid, and the subsequent binding of the $NodD_1$ protein to the conserved *nod* box sequence upstream of the *nod* operon (reviewed in Stacey, 1995).

The second activation pathway, reminiscent of the classical two-component signaling pathway, involves the NodVW proteins (reviewed in Loh *et al.*, 1996). NodV and NodW are essential for the nodulation of mungbean, cowpea, and siratro, which are alternative host plants of *B. japonicum*. In addition, NodW has also been demonstrated to be required for the full expression of the common nodulation genes in *B. japonicum*. This mediation of *nod* gene expression by NodW involves a series of phosphotransfer reactions which are initiated with the autophosphorylation of NodV in response to plant isoflavonoid signals. The phosphorylated NodV then phosphorylates the regulator protein by transferring the phosphoryl group to the conserved aspartate residue of NodW. This sequence of phosphotransfer reactions function to activate NodW, resulting in the mediation of *nod* gene transcription by phospho-NodW. The utilization of phosphorylation as a mode for *nod* gene activation appears to be unique for *B. japonicum* and plays a key role in *nod* gene activity and nodulation. For instance, recent work in our laboratory has shown that a NodW protein that contains an Asp70 to Asn70 mutation is no longer phosphorylated *in vitro* and *in vivo*. *B. japonicum* strains expressing these proteins demonstrate a drastic reduction in both *nod* gene activity and in their ability to nodulate mungbean.

A third component in the regulation of *nod* gene activity involves NolA, a member of the MerR-type family of regulatory proteins. NolA was first reported to be a genotype-specific nodulation gene. Further studies later showed that NolA, when expressed from a multi-copy plasmid in *B. japonicum*, shows a marked decrease in *nod* gene expression, suggesting that NolA may function as a repressor molecule. Recent results in our laboratory have now shown that NolA positively regulates its own expression, as well as the expression of *nod*D_2, thereby exerting its effect on *nod* gene transcription through regulating $NodD_2$. We have recently demonstrated that over-expression of $NodD_2$ results in a reduction of *nod* gene activity

(Garcia *et al.*, 1996). Microscopic analysis has also shown that NolA may also be involved not only in the early stages of nodulation through mediating the activity of gene regulation, but may also be involved in bacteroid development and maintenance. Using deletion mutants of *nol*A, electron microscopy of soybean nodules revealed a decrease in the number of infected cells at 14 days post-inoculation. Similarly, nodule phenotype of infected cowpea nodules showed a decrease in the number of infected cells, as well as a decrease in nitrogen fixation, as determined by an acetylene reduction assay. Hence, the third component of *nod* gene regulation in *Bradyrhizobium japonicum* appears to have a dual-gated role, necessary not only for the regulation of gene expression during the early stages of nodule morphogenesis and symbiosis, yet also functions as a house-keeping gene maintaining the integrity of the developing bacteroid.



FIGURE

Lipo-chitin oligosaccharides(LCO)

The protein products of the *nod* genes are responsible for the synthesis and transport of Nod Signals. These phytoactive molecules initiate plant growth responses on the roots of the host plant, resulting in the formation of a new organ, the nodule. *Bradyrhizobium japonicum* USDA110 produces a major LCO with a pentameric oligosaccharide backbone; additional minor compounds have also been identified. The N-acyl substitution at the non-reducing end is a C18:1 fatty acid(vaccenic acid), and 2-O-methyl fucose is linked at O-6

of the terminal reducing N-acetyl glucosamine (Carlson *et al.*, 1994). This single compound, when applied to the roots of *Glycine soja*, is capable of initiating the early responses seen at the onset of nodule morphogenesis. One such response is the induction of plant genes specifically expressed during the infection process. Several well characterized nodulins include leghemoglobin, uricase II, ENOD2, ENOD40, and ENOD55. The early nodulins represent a class of plant genes that are induced as early as 5 minutes post-inoculation with a single purified LCO. Certain ENODs, such as ENOD2, require the cooperative action of a mixture of LCOs. Recent work in our laboratory has been focused on dissecting the expression pattern of two early nodulins, ENOD2 and ENOD40 (Minami *et al.*, 1996a; Minami *et al.*, 1996b). Induced rapidly upon inoculation, these two genes represent two very distinct patterns of expression.

ENOD40 expression is rapidly induced in soybean roots upon inoculation with various LCOs, including LCOs which do not promote responses such as root hair deformation (HAD) or nodule initiation (NOI)--the signature responses of rhizobial infection (Kouchi and Hata, 1993). Interestingly, on *Glycine soja*, we were able to show that expression of ENOD40 does not require specific chemical substituents on the LCOs as previously thought, and that ENOD40 expression is inducible in a non-specific manner, with regard to LCO signaling. ENOD2 expression on the other hand does require the cooperative action of at least two LCO molecules and induction does appear to require a high degree of specificity. In addition to the requirement for two signal molecules, there is the added requirement that one of the compounds must be able to elicit a HAD or NOI response. These results led us to the paradox of why *B. japonicum* USDA110 produces a mixture of at least 4 Nod factors, when in fact HAD and NOI responses could be induced upon inoculation with a single compound. Microscopic analysis and *in situ* hybridization of inoculated roots have led us to the possibility that differentiation of nodule parenchyma does not occur in those primordia induced by a single molecule. Furthermore, it is apparent that multiple signals are required for the progression of nodule ontogeny, as is evidenced by the expression pattern of ENOD2. This has led us to the belief that there may in fact be at least two signaling events involved in the establishment of a successful nitrogen-fixing symbiosis, as proposed by Ardourel *et al.* (1). Indeed, much work remains to elucidate the signaling mechanism(s) involved in plant-microbe symbiosis, as well as uncovering the plant receptor(s) which recognizes the various signal molecules.

Future prospects

We are now beginning to understand the involvement of LCOs in establishing the limits of host-range specificity in a number of plant-microbe interactions, as well as to determine

the functions of the various *nod* gene products in the process of regulation and synthesis. However, we have only begun to scratch the surface as far as our understanding of signal recognition and transduction. To better understand the signaling mechanism, we are now focusing on understanding the regulation and involvement of several plant gene products involved in this process. Several laboratories have begun to make great strides in characterizing possible receptors for LCO compounds, yet much remains to be done to show their exact involvement in the process of nodulation and nitrogen fixation.

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Bacterial Collections for Studying Soil Bacterial Community

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In soil microbaial ecology, it is of the central importance to elucidate the composition of bacterial communities. Methods of analyzing a library of DNA molecules retrieved directly, without culturing bacteria, from natural environments to discover the uncultured bacteria are now developing. They were able to successfully detect novel bacteria (1, 2, 4, 5, 9, 11, 12). However, such culture-independent methods are suffering from efficiency of extraction of nucleic acid, PCR amplification, and cloning of genes among bacteria; and chimeric products could be present in mixed population of the PCR products (8, 10). Moreover, we cannot obtain organisms themselves by the culture-independent method for further investigation. For the reasons the culture method is still important.

We have constructed bacterial collections for studying bacterial community in soil. The collections were obtained by systematically isolating bacteria based on the time of appearance of colonies on DNB (100-fold dilution of nutrient broth) agar plates. At present we have the collections from paddy field (6), grassland (3, 7), forest land (13), and cultivated land. Here we outline our work about phylogenetic analysis of the collection from paddy field soil by 16S rRNA gene sequencing.

The paddy field soil bacteria was isolated as follows (6). 1 ml each of 10^5 dilution of soil suspension from 1 g of paddy field soil, was incubated with DNB agar on ten plates. Bacterial colonies were picked based on the time of the appearance. They were divided into four growth rate groups. The groups I, II, III and IV consisted of strains forming colonies within 30 h, 31 to 51 h, 52 to 114 h and 115 to 265 h, respectively. Thirty to fifty strains were obtained from each group.

In the present study, test strains were selected randomly from each growth rate group. For phylogenetic analysis, a 0.5 kb-DNA fragment encoding part of the 16S rRNA gene was amplified by PCR from genomic DNA of each strain, and sequenced. Based on the sequences, we made a phylogenetic tree (see cover picture).

The phylogenetic tree showed the existence of various bacteria. The isolates

belonged to various eubacterial groups including: low G+C gram-positive bacteria; high G+C gram-positive bacteria; Cytophaga/Flexibacter/Bacteroides group; α-Proteobacteria; β-Proteobacteria; and γ-Proteobacteria. More than half of them were related to known species in the DNA database with a sequence similarity of more than 95%, but no sequences matched perfectly those in the database. The results suggest that these strains can be affiliated with the known genera even if they are not identical to the known species. The similarity values of the other strains ranged from 89% to 94% for the most similar sequences in the database. It is possible that they are affiliated with new genera.

The collection included several distinct clusters of closely related strains, especially the clusters neighboring *Arthrobacter globiformis* and *Zoogloea ramigera*. Some strains within the clusters showed identical sequences with one another, and others showed at most a 5% difference. These sequence variations may correspond to the diversity among species or subspecies.

Although the strains from each growth rate group were affiliated with various phyla, some strains in the same growth rate groups often formed phylogenetic clusters. Many strains in group I and II, faster growers, neighbored *A. globiformis* or *Z. ramigera*. Several strains in group III, organisms of intermediate growth rate, made up dispersed clusters together with *Dermatophilus congolensis* in high G+C gram-positive group or with *Rhizomonas suberifaciens* in α-Proteobacteria group. Strains in group IV, slow growers, made up clusters together with *Caulobacter crescentus* or *Bradyrhizobium japonicum* in α-Proteobacteria group.

Correspondence of growth rate grouping to phylogeny, if it generally exists as suggested from our result, is important from the aspect of population dynamics and/or bacterial evolution and diversification.

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One Approach to Study Morphological Development in the Zygomycete Fungus *Phycomyces blakesleeanus*

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The zygomycetous fungus *Phycomyces blakesleeanus* is well characterized: because of gigantic sporangiophores and their sensitibity to blue light, to gravity, to mechanical stretches, and to the presence of nearby barriers (Bergman et al., 1969); and dynamic morphological changes in the sexual development which undergoes when opposite mating partners meet at the hyphal tip (Cerdá-Olmedo and Lipson, 1987). The phototropic response of the sporangiophore is dependent on asymmetric elongation at the growing zone of the sporangiophore (Castle, 1965). The mating response of the sexual organs is dependent on exactly normal formation of specific structures (Yamazaki and Ootaki, 1996). To establish both morphogenesis the deposition and synthesis of new cell wall will unquestionably required. Main components of the cell wall in zygomycete fungi are chitin and chitosan,which can be considered as deacetylated form of chitin and is a characteristic component in the cell wall of Zygomycetes (Ruiz-Herrera 1992). As chitosan is thought to be matricial components, chitin synthesis is likely to be a main target for supporting rigidity and structure to establish the growth and morphological development in this fungus.

Chitin is a ß (1,4)-linked polymer of N-acetylglucosamine (GlcNAc). The polymer can account for up to 25-30% of the cell wall in sporangiophores of *P. blakesleeanus* (Kreger 1954). Its synthesis is carried out by incorporation of GlcNAc units from UDP-activated GlcNAc in the reaction catalysed by chitin synthase (CHS): UDP-2-acetamido-2-deoxy-D-glucose: chitin 4-ß-acetamidodeoxy-D-glucosyltransferase (EC 2.4.1.16). Cell biological and biochemical studies have localized CHS activity to both chitosomes (Bracker et al. 1976) and plasma membranes (Duran et al. 1975), and have shown that there exist two types of enzymes: a zymogen type which requires partial proteolysis for activation *in vitro* (Cabib and Farkas 1971), and a non-zymogen type which does not require proteolytic activation (Orlean 1987).

Multiplicity of the genes encoding CHS is known in almost the filamentous fungi studied (Bowen et al. 1992) as well as in yeast *Saccharomyces cerevisiae* (Bulawa 1993). An attractive possibility is that each gene is temporally and spacially regulated in cell wall synthesis during cell growth and differentiation. In fact, functional analysis using gene disruptants has shown that certain gene has a special manner of expression responsible for cell morphology in *Aspergillus nidulans* (Motoyama et al. 1997). In the zygomycete fungus *P. blakesleeanus*, only one clone was identified so far; it belongs to class II-CHS (Miyazaki et al. 1993). It is pointed out that the increased chitin content and developmental complexity correlated with the finding that filamentous fungi had a larger number of CHS genes (Speckt et al. 1996). That is in case of *P. blakesleeanus* when thinking both the content of chitin and chitosan and the developmental complexity.

Two different primer sets newly designed were used in polymerase chain reactions to amplify the fragments of CHS genes from *P. blakesleeanus*. DNA-sequencing and alignment analysis of the deduced amino acid sequences showed the existence of ten different genes. Six different DNA fragments, designated *PbCHS1, PbCHS2, PbCHS3, PbCHS4, PbCHS5* and *PbCHS6* are identified in 250-bp products. From 350-bp products four different fragments, *PbCHS7, PbCHS8, PbCHS9* and *PbCHS10* were obtained. Clustal analysis suggested that while this fungus was thought not to have either class I- or III-CHS, class II- and IV-CHS were present as multiple form. *PbCHS1-4* were in class II and *PbCHS7-10* belonged to class IV. Interestingly two fragments, *PbCHS5* and *PbCHS6*, were located near CHS1 of *S. cerevisiae*, which is divided into non-class category. Only *PbCHS1, PbCHS2, PbCHS7* and *PbCHS8* genes were actively expressed in the young germlings cultured in the liquid medium. Northern analysis revealed that *PbCHS1, 2* and *PbCHS7* were transcribed as 3.2- and 4.7-kb mRNA in length, respectively. Transcriptional products from *PbCHS8* and *PbCHS10*, 8.2 kb mRNA in length, might suggest the existence of a unidentified type of CHS or the possibility of a multifunctional gene including CHS function. Further study, in particular to clarify the *in situ* localization of each protein and mRNA of the CHSs during morphogenesis is required. This effort can lead us to understand the mechanism on the developmental regulation of this fungus at molecular level.

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Comparative Studies of the Structure of Chloroplast DNA and Phylogenetic Relationships in the Genus *Asparagus* (Liliaceae)

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Sex differentiation in higher plants is a striking phenomenon. An extensive catalog of sexuality in 120,000 plant species indicates that hermaphrodites are very common (about 72%), while only 4% of angiosperms are dioecious (Yampolsky and Yampolsky, 1922). Since dioecy is sporadic, with occasional species being found in otherwise hermaphroditic genera, this breeding system might have evolved recurrently (Parker, 1990), perhaps by the developmental arrest of the inappropriate sex at an early stage of floral development in dioecious plant (Dellaporta and Calderon-Urrea, 1993).

Garden asparagus (*Asparagus officinalis* L.), an economically important horticultural

Fig. 1. Hermaphrodite (left), pistillate (middle) and staminate (right) flowers of *Asparagus* palnts.

crop, is a dioecious species (Fig. 1). Sexual dimorphism in this species is controlled by the genetic factors X and Y; female plants are homogametic (conventionally XX), while males are heterogametic (XY) for sex chromosomes (for review, see Bracale et al., 1991). Since the sex chromosomes are homomorphic (Löptien, 1979) and plants with the YY genotype are viable, the system for sex determination in *A. officinalis* appears to have evolved relatively recently (Dellaporta and Calderon-Urrea, 1993).

The genus *Asparagus* consists of 100-300 species and it is distributed mainly on dry land in the Old World (Bailey, 1944; Chittenden, 1956; Ohwi, 1965). The genus includes hermaphroditic and dioecious species with varying morphology. *Asparagus* is found as herbaceous perennials, tender woody shrubs and vines (Bailey, 1944). Moreover, *Asparagus* species were classified into four sections by Bailey (1944): Euasparagus, Asparagopsis, Kodiastigma and Myrsiphyllum. More recently, Clifford and Conran (1987) and Dahlgren et al. (1985) divided *Asparagus* (Asparagaceae) into three subgenera: Asparagus, Protasparagus and Myrsiphyllum. Although dioecious species were classified as the section Euasparagus and the genus Asparagus by Bailey (1944) and Dahlgren et al. (1985), respectively, there are some differences in these classifications: *A. scandens*, *A. plumosus*, *A. falcatus* were classified into section Asparagopsis and *A. virgatus* and *A. asparagoides* were classified into sections Kodiastigma and Myrsiphyllum, respectively, by Bailey (1944). By contrast, *A. plumosus* and *A. virgatus* were classified into Protasparagus, and *A. scandens* was classified into Myrsiphyllum with *A. asparagoides* by Clifford and Conran (1987). Previous cytological studies of *Asparagus* revealed the presence of polyploid series in this genus. However, correlations between numbers of chromosomes and phylogeny have not yet been described.

Systematic studies of variations in chloroplast DNA (ctDNA) have been relatively common in many groups of higher plants. To investigate the phylogenetic relationships in the genus *Asparagus*, we have cloned and constructed a physical map of asparagus ctDNA (Lee et al., 1996). From the restriction maps of their ctDNAs, eight species of *Asparagus* examined in this study, namely, *A. asparagoides*, *A. macowanii*, *A. scandens*, *A. virgatus*, *A. cochinchinensis*, *A. plumosus A. schoberioides*, *A. officinalis*, seemed to be very closely related. However, values of interspecific divergence (100 x p) ranged from 0.4 to 2.4 among the species. Previous authors have estimated ranges of values of 100 x p in several group of plants: 0.0 to 0.3 in *Zea*; 0.24 to 1.0 in *Aegilops/Triticum*; 0.0 to 2.7 in *Sorghum*; and 0.0 to 1.1 in *Bromus*. Compared to these groups, the various species of *Asparagus* had higher variation in their ctDNAs. Despite the higher degree of diversity among ctDNAs, only one deletion of about 1 kb was found in

the ctDNAs of *Asparagus*. In *Zea* and *Aegilops/Triticum*, four and fourteen deletions or insertions were reported, respectively. The lengths of the deletions in *Zea* were 80 to 250 bp and those in *Aegilops/Triticum* were 0.1 to 0.9 kbp. Therefore, it is possible that there are many more small deletions and insertions in the ctDNA of *Asparagus*.

The phylogenetic tree shows that the species of *Asparagus* examined in this study could be divided into two clusters, with *A. virgatus* and *A. plumosus* being separated from the other species. Clifford and Conran (1987) grouped *A. virgatus*, *A. plumosus* and *A. sprengeri* (*A. densiflorus*) together into the subgenus Protasparagus. However, *A. sprengeri* seems to belong to another group.

The ctDNAs of *A. sprengeri* and *A. falcatus* gave the same restriction patterns, which indicated that these species are very closely related. *A. falcatus* and *A. sprengeri* are tetraploid (2n=40) and hexaploid (2n=60), respectively. It is of great interest that the polyploid species of *Asparagus* were grouped in one cluster, and this grouping suggests that the polyploidy in this genus might have a single origin.

The dioecious species have been classified into the section Euasparagus (Bailey, 1944) and into the genus Asparagus (Dahlgren et al., 1985; Clifford and Conran, 1987). The dioecious species used in this study, namely, *A. officinalis*, *A. schoberioides* and *A. cochinchinensis*, were grouped into one cluster and appear, therefore, to be monophyletic. We also confirmed that these three species are monophyletic by RAPD analysis. The species are distributed in separate locates: *A. officinalis* is found in Europe and *A. schoberioides* and *A. cochinchinensis* are found in Asia. This distribution suggests the possibility that the origin of the dioecy in *Asparagus* was monophyletic and that these dioecious species have the same mechanism for sex determination. Additional characteristic, such as diversity of the nuclear DNA, and studies of many more species of *Asparagus* should provide further information about the taxonomy and evolution of this genus.

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Production and Analysis of Plants That Are Somatic Hybrids of Barley (*Hordeum vulgare* L.) and Carrot (*Daucus carota* L.)

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The goal of plant breeding is the construction of new genotypes by the introduction and the manipulation of genetic variations. The production of somatic hybrid plants by protoplast fusion is a useful method for the combination of genetic materials. Protoplast fusion can sometimes lead to the production of new genetic variants as a consequence of the recombination of nuclear and of cytoplasmic genomes. Many intra- and interspecific and several intergeneric somatic hybrid plants have been reported. Recently, asymmetric hybrids between remote species, for example interfamilial hybrid plants have been obtained by exploiting various systems for the selection of hybrids (Somers et al., 1986; Dudits et al., 1987; Kisaka and Kameya 1994; Kisaka et al., 1994).

Barley (*Hordeum vulgare* L.) is a crop plant that tolerates low- temperatures and salinity. To examine the possibility that these characteristics of barley might be transferable to other crops by protoplast fusion, we attempted to produce plants that were somatic hybrids of barley and carrot (*Daucus carota* L.), utilizing the low-temperature tolerance of barley for selection of hybrids. In the present report, we describe production and analysis of somatic hybrids of barley and carrot.

Effects of low-temperature treatment

When cells from 6-month-old carrot suspension cultures were plated on MS medium supplemented with 0.8% agar and incubated at 4℃ for various periods and then the calli were transferred to 25℃, the number of the regenerated calli decreased with increasing duration of the low-temperature treatment. Most of calli incubated at 4℃ for 5 weeks and 6 weeks were not regenerated. On the basis of the result, the low-temperature treatment for selection of hybrid calli consisted of incubation at 4℃ for 5 weeks after incubation for one month at 25℃ of fused cells.

Protoplast fusion and culture of fused cells

Protoplasts of carrot, isolated from cells in

suspension culture, and those of barley, isolated from young leaves were fused by electrofusion and cultured on MS medium supplemented with 5.0% (w/v) glucose, 1.0 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.5 mg/l Kinetine. After culture for one month at 25℃, the fused cells were transferred to MS medium supplemented with 1.0% (w/v) agar, 1.0 mg/l BAP (N6-benzylaminopurine) and 0.1 mg/l NAA (naphthaleneacetic acid) and then incubated at low temperature (4℃) for 5 weeks in darkness. The resultant calli were transferred to continuous light (4 W/m^2) at 25℃. When visible colonies had developed to about 1-2 mm in diameter, about 2,700 colonies were transferred to fresh medium. Three shoots were regenerated and these were transferred to rooting medium (MS hormone free) supplemented with 0.8% (w/v) agar. The three regenerated plants were potted in soil and designated no.1, no. 2 and no. 3.

The protoplasts of barley that had been isolated from young leaves failed to divide. The protoplasts of carrot that had been isolated from 6-month-old suspension cultures proliferated and formed colonies. However, about 1,400 colonies that had been incubated at 4℃ for 5 weeks failed to regenerate any shoots. Furthermore, no plants were obtained from protoplasts of either barley or carrot that were cultured under the same conditions without fusion treatment.

Analysis and characterization of the three regenerated plants

The somatic hybrid plants closely resembled carrot in morphology. Hybrid no. 1 had variegated green and white leaves and flowers, which developed without vernalization (Fig. 1). The morphology of the roots of the somatic hybrids was similar to that of roots of carrot. The flowers exhibited male sterility as did those of the parent strain of carrot.

Callus cultures induced from leaf segments of the regenerated plants and their parents were analyzed at the cytological and molecular levels. Cytological analysis revealed that the chromosome number of the regenerated plants was about 24, namely, significantly lower than the sum of the chromosome numbers (32) of the parents. Genomic DNA was analyzed by Southern hybridization with a non-radioactively labeled DNA fragment of the *rgp1* gene (Sano and Youssefian, 1991). The regenerated plants generated both a band specific for carrot (4.4 kbp) and a band specific for barley (3.6 kbp). Chloroplast (ct) and mitochondrial (mt) DNAs were also analyzed by Southern hybridization with fragments of ct DNA and mt DNA. The results of analysis of ct DNA with a non-radioactively labeled fragment of rice ct DNA of BamHI-8 as probe indicated that the regenerated plants yielded both bands specific for carrot (4.2 kbp and 2.2 kbp) and a band specific for barley (9.0 kbp). The regenerated plants also yielded a band specific for barley (4.4 kbp) and a unique band (8.6 kbp) when

the *Bam*HI-3 fragment of rice ct DNA was used as the probe. In the analysis of mt DNA, one of the regenerated plants (no. 1) yielded a novel band (9.0 kbp) that was not detected in the either analysis of parent when a fragment of *atp6* was used as the probe. These results indicated that the regenerated plants were somatic hybrids between barley and carrot. This study has been published in Theor. Appl. Genet. (Kisaka et al., 1997).

Appendix

Recentley, we examined the somatic hybrids between barley and carrot to determine whether or not the cold tolerance and salt tolerance of barley had been transferred to the samotic hybrids. As a result, one of the somatic hybrids (no. 2) was more tolerant to cold and NaCl than carrot, as was barley. Environmental stress such as cold and salinity have been recognized as major factors that limit crop productivity, and various attempts to breed environmental stress tolerant crop plants have been made. However, on optimum breeding strategy has yet been developed, as a consequence of our limited understanding of the mechanisms of environmental stress tolerance in higher plants. But we think that protoplast fusion is useful method for introduction the tolerance of environmental stress from tolerant plants to somatic hybrids.

Fig. 1 Plants of carrot (left), a somatic hybrid (middle) and barley (right).

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IAA-Inducible and *ETR1*-Like Genes Isolated from Cucumber Seedlings and Their Possible Involvement in the Gravity-Regulated Formation of Peg and Hypocotyl Hook

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In a horizontally germinated cucumber seedling, hypocotyl develops a hook and a peg. The peg formed on the concave side of the bending hypocotyl and arching hook are advantageous for the emergence of the cotyledons and plumule from the seed coat.

It has been shown that gravity regulates the formation of both hook and peg in cucumber seedlings (Takahashi and Suge 1988, 1994). Hypocotyls of mature embryos in cucumber seeds are straight, and the orientation of hook formation is determined by direction of gravity when they germinate. The peg usually develops only on the lower basal region of the hypocotyl (on the concave side of arching hook) when seeds germinate in a horizontal position. On the contrary, seedlings germinated in a vertical position scarcely form hook at the early stage of growth. In addition, seedlings in a vertical position do not develop a peg or develop two pegs on both sides of the straight hypocotyls showing the bilaternal symmetric growth.

Auxin-inducible genes

When cucumber seeds were germinated carefully in a vertical position, the seedlings exhibited straight growth without formation of a hypocotyl hook and failed to develop a protuberant peg. In this condition, exogenous IAA could induce a peg-like protuberance (Takahashi and Suge 1988). In addition, application of an auxin transport inhibitor (TIBA) showed different effects on peg formation in horizontally germinated seedlings, depending upon the concentration used. TIBA at 10^{-4} M inhibited peg development, but at 10^{-5} M two distinct pegs were induced on both the lower and upper sides of the horizontally growing hypocotyl. TIBA at the concentrations of 10^{-4} M and 10^{-5} M inhibited hook formation. These results suggested that redistribution of auxin is involved in the development of both peg and hook.

Auxin is known to induce rapid expression of genes; that is, expression of *Aux/IAA* gene family and *SAUR* (small auxin up-regulated RNAs) gene family are induced within several

minutes following auxin application. A number of *Aux/IAA* genes have been isolated from pea, soybean, mungbean, and Arabidopsis. Comparison of each gene shows that *Aux/IAA* genes have four conserved regions (domain I to domain IV) (Abel et al. 1994). To obtain similar genes for the study of the gravity-regulated morphogenesis, we performed RT-PCR with a forward primer designed from amino acid sequences TELRLGL in domain I, and a reverse primer designed from amino acid sequences KRLRIMK in domain IV including a nuclear localization signal sequence. By this means, we isolated three PCR products, 483 bp (*CsAux22*), 540 bp (*CsIAA4*), and 795 bp (*CsIAA8*) from cucumber seedlings. The deduced amino acid sequences in the amplified region of *CsAux22*, *CsIAA4*, and *CsIAA8* showed sequence similarity to *Aux22* (47%) of soybean (Ainley et al. 1988), *IAA4* (59%) and *IAA8* (51%) of Arabidopsis (Abel et al. 1995), respectively. Responsiveness of these genes to auxin was examined with hypocotyl sections of 3-days-old etiolated seedlings. Accumulation of *CsAux22* and *CsIAA4* mRNA in the sections decreased by auxin starvation for 2 h and remained steady-state amount thereafter at least for further 2 h without addition of exogenous IAA. Treatment of the hypocotyl sections for 2 h with IAA at 10^{-7} to 10^{-4} M following the 2 h auxin starvation induced mRNA accumulation of *CsAux22* and *CsIAA4* genes. *CsIAA8* did not show an apparent response to auxin in this experiment. Further studies on the expression of auxin-regulated genes may be clue to clarify the mechanism for auxin action in the gravity-regulated morphogenesis of cucumber seedlings.

ETR1-like genes

Peg development on the lower side of the transition zone is inhibited by inhibitors of ethylene biosynthesis and ethylene action (Takahashi and Suge 1988). The ethylene inhibitors also inhibited the formation of hypocotyl hook in cucumber seedlings (Takahashi and Suge 1988).

The *ETR1* gene isolated from Arabidopsis codes for an ethylene receptor (reviewed by Bleecker and Schaller 1996). To understand ethylene involvement in the gravity-regulated formation of hook and peg, we have attempted PCR cloning of the *ETR1* gene from cucumber. Several mutations in the amino-terminal hydrophobic region of the *ETR1* gene in Arabidopsis confer ethylene-insensitivity on plants with a dominant inheritance (Chang et al. 1993). A forward primer was designed from amino acid sequences VVSCATA containing one of these mutations (Ala^{102} to Thr in *etr1-2*). The carboxyl-terminal half of the ETR1 contains similar sequences to the histidine kinase domains and response regulator domains of signal transducers known as the two-components system. A reverse primer was designed from amino acid sequences MNEHMRT containing the conserved histidine residue that is the site of

autophosphorylation. Using those primers, we isolated two partial-length cDNAs similar to *ETR1* gene from cucumber with RT-PCR. In analysis of their mRNA accumulation with northern blotting, we found that the accumulation of mRNA of *ETR1*-like genes in the inner part of arching hypocotyl was much less than that in the outer part of the arching hypocotyl. In addition, their mRNA in the apical part of hypocotyl accumulated during hook development and decreased during hook opening. These results suggest that the expression of ethylene receptor genes is modulated during gravity-regulated morphogenesis in cucumber seedlings.

Schwark and Schierle (1992) proposed a model of an interaction between ethylene and auxin in regulating hook maintenance of *Phaseolus vulgaris* L. Briefly, auxin induces ethylene synthesis, and ethylene inhibits transport of auxin. These distinct effects somehow lead to an inhomogenous distribution of auxin and ethylene, and cause differential cell growth. It would be worthwhile to investigate whether this model is accommodated to the gravity-regulated formation of hook and peg in cucumber seedlings.

In future, transgenic cucumber plants that have ectopic expression of *ETR1*-like genes will reveal the significance of regulated expression of *ETR1*-like genes. In addition, a missense mutation in either of two predicted transmembrane domains in ethylene receptor genes of Arabidopsis and tomato confer dominant ethylene insensitivity to wild-type plants (Chang et al. 1993, Hua et al. 1995, Wilkinson et al. 1995). Integration of similar missense-mutated *ETR1*-like genes into cucumber may confer ethylene insensitivity to cucumber plant. These ethylene insensitive transgenic cucumber plants will be useful for the study of ethylene signaling pathway in the development of peg and hook in cucumber seedlings.

Space experiments

We are currently preparing for a spaceflight experiment on the gravimorphogenesis of cucumber seedlings. Analysing the expression of the auxin-inducible and *ETR1*-like genes in cucumber seedlings under microgravity conditions may be useful for verifying the hypothesis for the mechanism of the gravimorphogenesis of cucumber seedlings.

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Relationship Between the Expression of *EXGT* Gene and Differential Growth in Hydrotropically Responding Roots of *Ageotropum* Pea

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Roots exhibit a positive tropistic response to a moisture gradient or a sorbitol-induced water potential gradient at the root cap (Takahashi and Scott 1993, Takano et al. 1995). Root hydrotropism is induced by asymmetric application of sorbitol agar block to the root cap, which shows root curvature away from the sorbitol source (Takano et al. 1995). Water-potential gradient as small as 0.5 MPa m^{-1} at the root cap has been shown to induce the curvature associated with the hydrotropic response (Takano et al. 1995). This curvature occurs due to a differential change in cell wall extensibility in elongation zone of a hydrotropically responding root (Hirasawa et al. 1997), confirming that hydrotropic curvature results from the differential cell elongation growth in the elongation zone.

Endo-xyloglucan transferase (EXGT) has been considered to play an important role in cell extention growth, which cleaves xyloglucan polymers internally and ligates the newly genelated reducing end to another xyloglucan chain (Fanutti et al. 1993, Farkas et al. 1992, Fry et al. 1992, Nishitani 1995, Nishitani and Tominaga 1992, Smith and Fry 1991). Because xyloglucans are thought to cross-link cellulose microfibrils in the plant cell wall (Fry 1989, Hayashi 1989, Maccann et al. 1990, Passioura and Fry 1992), EXGT activity may be critical in determining properties of the walls of differentially elongating cells in the elongation zone of hydrotropically responding roots. Recently, *EXGT* gene was isolated and shown to be involved in the cell elongation growth (Nishitani 1995).

To clarify the differential growth of hydrotropically responding roots, therefore, we isolated *EXGT* gene from pea roots and examined its role in root hydrotropism. The

roots of the pea mutant, *ageotropum*, were used in this study because the hydrotropic response of this mutant is unimpeded by gravitropic interference (Jaffe et al. 1985, Takahashi and Suge 1991, Takano et al. 1995).

We obtained a partial cDNA of *Ps-EXGT* from *ageotropum* pea roots with RT-PCR. The partial cDNA was 632 bp and showed 90% homology to the deduced amino acid sequence previously reported for soybean *EXGT* gene in amplified region. Using this partial fragment of *Ps-EXGT* gene as a probe, we then examined the expression of the *Ps-EXGT* gene in *ageptropum* pea roots.

First, we tested whether the expression of the isolated *Ps-EXGT* is coorelated with elongation growth because *EXGT* is consisting a multi-gene family and not all of them directly regulate cell elongation. Seedlings of *ageotropum* pea were grown in the presense of 1 MPa sorbitol or without such water stress. The treatment with 1 MPa sorbitol obviously inhibited the root growth as compared with the control roots. At 0, 1, 3 and 6 h following the treatment of water stress, 40 primary roots were harvested for RNA extraxtion. Northern blot analysis showed that the accumulation of *Ps-EXGT* mRNA was more abundant in the fast growing roots than the slow growing roots at all time points. These results indicate that the expression of *Ps-EXGT* gene correlates with elongation growth in *ageotropum* pea roots.

We next examined whether the *Ps-EXGT* gene is differentially expressed in the hydrotropically responding roots of *ageotropum* pea. Apical 8 mm length of primary roots including the elongation region were obtained at 0, 1, 2, 3, 4, and 8 h after the asymmetric application of sorbitol agar block to the root cap and longitudinally cut into halves of the sorbitol-treated side and non-treated side for RNA extraction. Fourty roots were used for each sample. A time-course study by northern blotting revealed that *Ps-EXGT* gene is differentially expressed in response to a moisture gradient. We have shown that gradient in water potential applied to the root cap cause rhythmic oscillating movement in hydrotropically responding root (Takano et al. 1995). The differential expression of *Ps-EXGT* gene appeared to account for the rhythmic oscillating movement of the hydrotropically responding roots.

The present results supported that the change in cell wall extensibility is responsible for the differential growth of hydrotropically responding roots (Hirasawa et al. 1997), in which EXGT plays an important role. We are now be able to study factors responsible for causing the differential expression of *Ps-EXGT* gene in roots. Because the sensory apparatus resides in the root cap and because calcium ion in the root cap appears to play a role in the signal transduction of hydrostimulus, some physiological changes due to a water potential gradient in the root cap may cause the differential expression of *Ps-EXGT* gene, which ultimately leads to the hydrotropic bending of roots.

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Cover pictures

Front cover: The dioecious species, *Asparagus officinalis*, *A. schoberioides* and *A. cochinchinensis*, were grouped into one cluster and appeared to be monophyletic (see the paper by A. Kanno; p. 12-15). Photograp shows *Asparagus* plant with hermaphrodite flowers.

Back cover: Phylogenetic tree including strains of bacteria from the paddy soil collecion. The growth rate groups (see text) are indicated in parentheses after the strain names. The scale bar represents substitutions per nucleotide position (see the paper by H. Mitsui and T. Hattori; p. 6-8).

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